UNIVERSITY OF PENNSYLVANIA

LACTIC ACID FORMATION IN LIVER TISSUE IN VITRO

PHYLLIS A. BOTT

A THESIS

IN PHYSIOLOGICAL CHEMISTRY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

PHILADELPHIA
1930

LACTIC ACID FORMATION IN LIVER TISSUE IN VITRO

A THESIS

IN PHYSIOLOGICAL CHEMISTRY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF PENNSYLVANIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PHYLLIS A. BOTT

PHILADELPHIA

1930

R. UNIV.
BIBLIOTHEEK
LEIDEN

Introduction

A survey of the literature suggests that all tissues in the surviving condition have the ability to form lactic acid. Numerous studies have been carried out on muscle which is of peculiar interest because of its capacity to form lactic acid as a result of contraction. Other investigations have been made on different tissues by Irving (1928), Schmitz and Chrometzka (1925), Himwich and Jacobson (1927), and others. A considerable amount of information has also been gained by those who are interested in comparing the lactic acid formation of normal tissue with that of cancer tissue. Warburg and his associates (1924), Fahrig (1927), Loebel (1925), and Murphy and Hawkins (1928) have made studies of this kind.

The modern view of the carbohydrate transfer in the body (muscle glycogen → blood lactic acid → liver glycogen → blood sugar → muscle glycogen) emphasizes what is perhaps the most important cycle for maintaining the stores of muscle glycogen but does not take into consideration the intrinsic metabolism of the liver itself. The early perfusion experiments of Embden and his associates (1912[r]) and of Harada (1927), as well as various *in vitro* experiments of other investigators suggest that liver has the power to form lactic acid. In sharp contrast with these findings are those of Simpson and Macleod (1927) who conclude that liver does form lactic acid.

The work to be presented in this paper demonstrates that lactic acid formation can take place in surviving liver and that it is greatly influenced by certain factors. It explains to some degree the lack of agreement between the findings of various investigators.

Experimental Procedures

Tissues from various animals were used. A few experiments were performed in which rats, dogs, and pigeons were employed, but for the most part tissues were taken from rabbits which were from three to six months old. Except where otherwise designated, the animals were given as much food as they would eat for a few

days previous to the time of the experiment. Fasting animals, when used, were given water daily by stomach tube. Liver or muscle was removed under amytal anesthesia or after stunning the animal. The tissue was worked up as quickly as possible after its removal, the exact procedure varying for different types of experiments. Five or ten gram samples were used for each different set of determinations. When rats or pigeons were used, it was found necessary to use the hash produced by grinding and mixing the livers of several animals.

The lactic acid formation of tissue suspensions was studied following various kinds of treatment including cooling, freezing by different means, grinding, and slicing. Either 2 per cent bicarbonate or bicarbonate-Ringer's solution as described by Rosenthal (1929[2]) was used as the suspension fluid. Lactic acid determinations were run by the method of Friedemann, Cotonio, and Shaffer (1927), (using $KMnO_4 + H_2SO_4$) or by the Friedemann and Kendall (1929) modification (using $MnO_2 + H_3PO_4$). Lactic acid determinations on standard solutions of the acid or the zinc salt yielded results 98 ± 2 per cent of the theoretical value. One thousandth normal iodine was used in the titration, and 0.5–to 1.5 mg. of lactic acid were taken for each determination. It might be mentioned here that if the determination is run carefully, the permanganate oxidation is reliable and easy to carry out on pure solutions of lactic acid. The point at which oxidation of the lactic acid is complete is clearly marked by the appearance of a brown turbidity of manganese dioxide. For several reasons the use of colloidal manganese dioxide as an oxidizing agent together with phosphoric acid seems less simple in spite of the fact that the oxidizing agent need not be added as carefully to the lactic acid solution. When the colloidal solution of manganese dioxide has been kept for some time the aggregates become large enough to cause occasional clogging of the capillary tips of the funnel tubes. It is also difficult to tell when oxidation is complete when using the colloidal manganese dioxide solution, as a gray precipitate forms before a sufficient amount has been added. A large excess of the oxidizing agent seems to be necessary to provide complete oxidation. Nevertheless when determinations are being made on certain tissue

extracts, the Friedemann and Kendall method appears to be more accurate, yielding lower results than the Friedemann, Cotonio and Shaffer procedure.

In a number of experiments lactic acid was determined on extracts of tissue prepared by a method commonly used at present which involves the Schenk and Van Slyke precipitations. This involved the precipitation of protein by mercuric chloride from a hydrochloric acid solution, and the removal of sugar by copper sulfate and calcium hydroxide suspension. The quantities of Schenk reagents employed were such that the concentration of hydrochloric acid in the total volume was approximately 2 per cent, that of the mercuric chloride was 2.5 per cent, while the final volume of the solution was 10 or more times the weight of tissue used. The precipitate obtained in this way was always allowed to stand over night at 3° before filtering.

The livers of the well-fed animals were so rich in glycogen that the protein-free filtrate, especially when the livers had been ground, was turbid with colloidal glycogen which prevented the flocculation of mercuric sulfide on the addition of hydrogen sulfide. In consequence, it was impossible to filter off the mercuric sulfide. The most trouble was experienced with livers of rabbits which appear to contain more glycogen than those of other animals. Riesser and Hansen (1926) who experienced the same difficulty with liver filtrates, were able to bring down the colloidal mass by using large amounts of mercury. This did not always prove sufficient, in our experience, and we resorted to boiling the solution for a minute or two with the addition of a pinch of sodium sulfate after saturating with hydrogen sulfide. The solution was cooled under the tap as soon as flocculation was complete. The slight loss in weight (less than 1 per cent of total) due to the heating was neglected in the calculations. This loss was ascertained by weighting the flask before and after heating. Liver lactic acid content as determined on filtrates of this kind and on trichloracetic acid filtrates in which no heating was necessary agreed as well as did the various individual determinations on copper filtrates.

The customary procedure for removing reducing substances from tissue filtrates is that recommended for this purpose by

Clausen (1922). However, such amounts of copper sulfate and calcium hydroxide reagents as are generally used for blood or tissue filtrates were inadequate for the removal of reducing substances from the liver filtrates of the type described. This was especially true if the solutions had been heated, as a large part of the higher carbohydrate probably hydrolyzed during this treatment. It was found necessary to use 35 cc. of 10 per cent copper sulfate solution and 40 cc. of a 5 per cent suspension of calcium hydroxide, for every 20 cc. aliquot portion of neutralized protein-free filtrate (corresponding to about 1.6 grams of liver). These amounts were determined by increasing the volume of reagents used in repeated trials, until the resulting filtrates no longer reduced Benedict's reagent. Since it was impossible to predict the exact amount of carbohydrate which might be present, these large quantities of reagents were used routinely where the copper-lime method was employed.

When lactic acid was determined on copper filtrates from liver prepared as above it was noticed that in many cases as the end-point of the titration was approached the color appeared and faded rapidly and a considerable additional titration was necessary to produce a color which persisted for 30 seconds. The effect was that which would be produced by the presence of material which dissociated from bisulfite slightly less readily than does acetaldehyde. The fading was more pronounced when permanganate and sulfuric acid were used for oxidation. These observations indicated that some interfering substance was present in liver, and not entirely removed by the copper-lime treatment. It was, therefore, decided that an investigation of a method involving ether extraction should be made. Efficient continuous extractors of convenient capacity were obtained for this purpose. Forty cc. of lactic acid solution were placed in an extractor, the solution saturated with ammonium sulfate, 6 drops of concentrated sulfuric acid were added, and the solution was extracted with 100 cc. of ether. Extraction was continued for various periods of time at such a speed that ether dropped from the condensers in an almost continuous stream.

When extraction was discontinued evaporation was carried out as described by Clausen (1922) except that it was not found

necessary to evaporate "almost to dryness". After evaporation of the ether, the neutralized aqueous solutions, which were then transferred to 15 cc. beakers, were heated on a water bath for about 45 minutes during which time about half of the water evaporated, leaving approximately 5 cc. of solution. This was transferred to a 50 cc. volumetric flask and made up to volume. As a rule 10 cc. aliquots served for lactic acid determinations.

Anhydrous ether containing only 0.5 per cent of alcohol was used for extraction. Blanks were run on this ether, evaporating with water as described above. The 10 cc. aliquots produced blanks no larger than those always obtained with the reagents used in the lactic acid determination itself (0.3 cc. of 0.001 N iodine). If ordinary U.S.P. ether was used, a high blank (1.1 to 1.4 cc. of 0.001 N iodine) was obtained, but this could be reduced to about 0.4 cc. by shaking the ether immediately before using, with dilute alkaline potassium permanganate solution, and then twice with small portions of water.

Table I shows the recoveries of lactic acid after various periods of ether extraction. The extraction of such amounts as were studied (0.6 to 44 mg.) was complete within 2.5 hours. However, as an extra precaution, solutions from tissues were always extracted for at least three hours. There is evidently some danger in extracting for very long periods of time (as for example, 30 hours as recommended by Embden (1912[1]) for more concentrated extracts), because on keeping the acid aqueous solution in contact with ether for many hours, the apparent lactic acid content was sometimes raised. This was true even when the solutions were kept overnight in the refrigerator.

Table II shows the recovery of added lactic acid from suspensions of hashed liver as determined on copper-lime filtrates and on ether extracts. In calculating the results total volumes were calculated on the basis of the volumes of material used, the specific gravity of liver being assumed to be one. The recovery is shown to vary from 93 to 102 per cent after ether extraction. In two experiments (40 and 53), recovery after copper-lime treatment varies between 96 and 107 per cent. Experiment 38 shows that very poor results may sometimes be obtained with the copper-lime procedure.

TABLE I

RECOVERY OF LACTIC ACID BY ETHER EXTRACTION

DURATION OF EXTRACTION	AMT. LACTIC ACID TAKEN (BY WT. OF LACTATE)	AMT. LACTIC ACID TAKEN (DETERNED. ON SOL. BEFORE EXTRACTION)	AMT. LACTIC ACID RECOVERED (DETERNED. ON SOL. AFTER EXTRACTION)	RECOVERY BY EXTRACTION
HRS.	MGS.	MGS.	MGS.	PER CENT
8.0		0.62	0.62	100
4.0		0.63	0.63	100
4.0		1.89	1.93	102
2.5		1.26	1.26	100
2.5	1.672	1.63	1.65	101
2.5	3.344	3.28	3.30	100
2.5	22.080	21.73	21.67	100
2.5	22.080	21.73	21.45	99
2.5	44.160	43.47	43.55	100
2.5	44.160	43.47	43.55	100
2.0		1.26	1.24	98
2.0		1.28	1.25	98
2.0		1.28	1.23	96
2.0	9.837	9.75	9.15	94
2.0	9.837	9.75	9.33	95
1.5		1.28	1.05	81
1.5		1.28	1.19	93
1.5	3.451	3.34	2.73	82
1.5	3.451	3.34	2.90	89
1.0	1.440	1.38	1.16	84
1.0	3.451	3.34	2.75	82
1.0	3.451	3.34	2.70	81

An examination of the data in Tables II and III discloses that, in cases where the liver lactic acid is high, the lactic acid content of the tissue suspension as determined on copper filtrates is often higher than that determined on ether extracts. This is especially true when permanganate and sulfuric acid are used, and is clearly demonstrated in Table III where figures for incubated tissue as as well as fresh tissue are given. These higher values obtained on copper filtrates are usually associated with fading end-points, and suggest the presence of interfering substances other than glucose, since the solutions did not reduce Benedict's reagent. The copper-lime treatment may be somewhat effective, but not to a constant extent, in removing the interfering substance. In experiments 33 and 35 of Table III, results on copper filtrates

TABLE II

RECOVERY OF ADDED LACTIC ACID FROM SUSPENSIONS OF LIVER HASH

Results are Expressed as Mgs. per 100 Grams of Fresh Liver

EXPT. NO.	LIVER	LIVER + ADDED L. A.	L. A. ADDED	L. A. RECOVERED	PER CENT RECOVERY	METHOD
38	93	98	14	5	35	Cu-lime, MnO_2*
	95	98	14	3	21	Cu-lime, $KMnO_4$†
	75	88	14	13	93	Ether, MnO_2
	75	88	14	13	93	Ether, $KMnO_4$
40	23	66	43	43	100	
	25	71	43	46	107	Order as above
	28	69	43	41	95	
	28	70	43	42	97	
53	106	154	50	48	96	
	107	156	50	49	98	Order as above
	93	144	50	51	102	
	93	143	50	50	100	

* MnO_2 always used with H_3PO_4.
† $KMnO_4$ always used with H_2SO_4.

TABLE III

Lactic Acid Formation in Liver Hash

Results are Expressed as Mgs. Lactic Acid per 100 Grams of Fresh Liver

EXPT. NO.	CONDITION OF ANIMAL	INITIAL OR FINAL	CU FILTRATE		ETHER EXTRACT	
			$KMnO_4$ H_2SO_4	MnO_2 H_3PO_4	$KMnO_4$ H_2SO_4	MnO_2 H_3PO_4
24	Rabbit Well-fed Amytal	Initial	73*			
		Final	139*			
31	Rabbit Well-fed Amytal	Initial			107	
		Initial			111	
		Final			180	
		Final			177	
32	Rabbit Fasted 2 days Amytal	Initial	42		44	
		Initial	50		40	
		Final	100		90	
		Final	99		83	
33	Rabbit Well-fed Amytal	Initial	162*	154*		
		Initial	179	167	148	149
		Initial	165	153	149	150
		Final	308	252	256	256
34	Rats Well-fed Amytal	Initial	72	71	77	
		Initial	76	71		76
		Final	139	137	134	
		Final	140	140		134

35	Rabbit Well-fed Stunned	Initial Initial Final	122 180† 306	116 219† 303	99 101† 300	99 98† 303
36	Pigeons Well-fed Decapitated	Initial Final			72 161	
37	Rabbit Well-fed Amytal	Initial Final Final Final	108 185 198 (4 hrs. at 37°) 153 (2 hrs. at 25°)	97 193	97 177	97 177
38	Rabbit Well-fed Stunned	Initial Final	95 189	93 183	75 185	75 186
39	Rabbit Well-fed Stunned	Initial	152	114	108	108
40	Rabbit Fasted 10 days Amytal	Initial Final	25	23	28 65	28 65

* Proteins precipitated with trichloracetic acid.
† Frozen with liquid air.

from two samples of hash of the same liver were extremely erratic, whereas the ether extracts yielded consistent results. Even when 2 portions of the same protein-free filtrate were treated separately with copper and lime there was sometimes a great discrepancy between the results of lactic acid determinations carried out on the two copper filtrates. This may account for the odd results of experiment 38, Table II.

Acetone was apparently not responsible for the poor end-points. Preliminary distillation, as suggested by Friedemann, Cotonio, and Shaffer for the removal of volatile ketones and aldehydes, did not improve the end-points nor lower the apparent lactic acid values. Acetone would also have been extracted by ether, but the end-points of determinations on ether extracts were always sharp.

Copper filtrates from livers of fasting animals sometimes yielded less lactic acid than ether extracts. Here there was evidently less interfering material in the liver extract and there appeared to be some loss of lactic acid in the voluminous copper hydroxide precipitate necessary for complete removal of carbohydrate. In view of these observations, it was decided that the copper-lime method too often gave values on liver filtrates which were not reliable. Therefore, in the later experiments ether extraction was used almost exclusively, both methods of oxidation being used successfully.

It was not found necessary to use ether extraction on filtrates from muscle. Four cc. of copper sulfate solution and 4 cc. of calcium hydroxide suspension were ample to remove the sugar from 20 cc. of muscle filtrate. Good end-points were obtained when lactic acid was determined on the copper filtrates, and the results of these determinations agreed very closely with those on ether extracts.

Lactic Acid Formation in Liver

In the absence of oxygen considerable lactic acid formation occurs in intact striated muscle as well as in muscle hash or press juice. Meyerhof has shown (1926[1]) that carefully prepared muscle extract may form lactic acid readily if glycogen and

phosphate are added. Hashing, freezing, and pulverizing do not appear to interfere much with the formation of lactic acid by striated muscle. The ease with which lactic acid may be shown to form in supensions of striated muscle tissue prepared in different ways has led various investigators to use similar procedures for the study of lactic acid formation in other tissues. The results have been to a considerable extent erroneous. Warburg (1923) observed that glycolysis by tumor tissue is diminished by any procedure which causes the cells to be damaged. In the experiments recorded below various experimental procedures have been compared. They demonstrate the great importance of intact cell structure for lactic acid formation in liver.

Lactic Acid Formation in Liver Hash

In these experiments liver was taken from the animal after removal of the gall bladder, dropped into a beaker which was immersed in a mixture of ice and salt, and after a few minutes' cooling, was put through a meat grinder in a cold room at 3°. The hash was weighed to the nearest centigram in previously cooled and weighed flasks containing a volume of 2 per cent bicarbonate solution approximately equal to the volume of tissue used. After weighing, those samples which were to be used for the initial lactic acid content of the liver were precipitated. Allowing the hash to extract in the cold room for an hour before precipitation did not materially change results. By means of a water pump, air was removed from the flasks containing samples which were to be incubated. After incubation the proteins were precipitated and copper-lime filtrates or ether extracts were prepared as previously described.

In this series of experiments the routine procedure was to incubate at 25° for 2 hours and then at 37.5° for 2 hours. The period at 25° was included to permit the action of enzymes which might be unstable at higher temperatures. It seems, however, that this procedure was unnecessary, since practically the same amount of lactic acid production was obtained by simply incubating for 2 hours at 37.5° (for example, see Table VI, Experiment 42).

The results shown in Table III, indicate, in general, that the amount of lactic acid per unit weight of tissue hash about doubles during the incubation, the resulting lactic acid figures for livers of well-fed animals being much higher than those from fasting animals. Before incubation the lactic acid content of livers of well-fed animals ranged from about 70 to 150 mg. per cent, while after incubation it lay between 134 and 300 mg. per cent. The maximum value (300 mg. per cent) obtained, showed an increase in lactic acid during the incubation, of 200 mg. per 100 grams of tissue. These results are similar to those of Riesser and Hansen (1926), who incubated rabbit liver pulp at 43 to 45° and obtained final values from 157 to 302 mg. per cent as compared with initial values of from 76 to 122 mg. per cent. Krause (1927), using much the same procedure with the livers of dogs and rabbits, obtained similar results. For their lactic acid determinations these investigators used the Hirsh-Kaufman (1924) and Embden (1925) methods, involving the precipitation of proteins by the Schenk procedure, removal of sugar by the copper-lime method, and finally, determination of lactic acid by the v. Fürth and Charnass method. Their lactic acid figures may, therefore, be subject to the same criticisms as are our copper filtrate data. The v. Fürth and Charnass method involves the direct titration of the excess bisulfite in acid solution while the Friedemann, Cotonio, and Shaffer method makes use of the titration of sulfite released from the aldehyde-sulfite complex by the addition of $NaHCO_3$. On account of the difference in the procedures it is possible that interfering substances might affect the two determinations differently. Some of our Friedemann, Cotonio, and Shaffer determinations on copper filtrates were, therefore, checked by the v. Fürth and Charnass (1910) method. The agreement between lactic acid contents as determined by the two methods was as good as that of individual determinations by the Friedemann, Cotonio, and Shaffer method.

To show more conclusively that lactic acid was actually formed during the period of incubation, lactic acid was isolated in an experiment on liver hash. Liver from a large dog was used, and isolation was carried out as described by Embden (1912[1]). An amount of zinc lactate was isolated from the fresh liver which was equiv-

alent to 18 mg. of lactic acid per 100 grams of tissue. After incubation *in vacuo* with bicarbonate solution, the zinc lactate isolated was equivalent to 117 mg. per cent of lactic acid.

It is interesting to note that the lactic acid content of livers of well-fed animals (generally 70–150 mg. per cent) is higher than that of fasting animals (20–50 mg. per cent). Cori (1925) observed that while the livers of normal mice contained a higher concentration of lactic acid than livers of fasting mice, this relationship did not hold true with muscle. As the blood lactic acid level is about the same in well-fed and fasting animals, it appears that with high carbohydrate stores in the body the liver lactic acid is higher than that of blood or muscle. Liver of fasted animals produced a smaller amount of lactic acid during incubation than did that of well-nourished animals. This may be due to the smaller amount of carbohydrate available, or to other factors. It is possible that fasting causes a decreased capacity for lactic acid formation as well as a depletion of the carbohydrate stores. Rosenthal (1929[2]) makes a suggestion of this kind with regard to "extra fermentation" in liver.

Comparison of the Lactic Acid Formation in Hashed and Sliced Liver and Muscle

To determine whether hashing with the resulting damage to a large proportion of the liver tissue interferes with the capacity of liver to form lactic acid, comparisons were made of lactic acid formation in liver hash and slices. Two similar experiments were carried out with muscle. In Table IV are presented data which represent the lactic acid concentration before and after the incubation of liver and muscle prepared by slicing or hashing. Except where otherwise designated, the hash was prepared by passing the tissue through a meat chopper. To prepare the fine hash, the tissue was passed through the chopper several times. This produced a pulp in the case of the liver, but the muscle, being more fibrous, hung together in fairly well-mashed shreds. Because of the large amount of material necessary for the determinations as usually run (5 grams) and the desirability of getting the tissue ready as quickly as possible, the slices were

TABLE IV

A Comparison of the Lactic Acid Formation in Hashed and Sliced Tissue of Rabbits

(Bicarbonate-Ringer's at 37.5°C)

Expt. No.	Temp. during preparation	Initial	Final		Increase in slices / increase in hash
			Hashed	Sliced	
		Lactic acid mg. per 100 grams of fresh tissue			
		Liver 120′ N_2			
52	25	117	168*	225	2.08
		Liver 30′ O_2 120′ N_2			
44	3	114	161*	481	8.00
46	3		181	285	
49	3		164	259	
50	25	111	222	372	2.35
51	25	128	238	406	2.53
		Muscle 120′ Vacuum			
46	3	297	846*	821	0.95
		Muscle 120′ N_2			
49	3	581	947*	1012	1.18

* Ground several times.

not cut as thin as is customary for the Warburg (1923) manometric measurements. The average slice thickness was about one millimeter. Slices were dropped in turn, into the various flasks (containing 45 cc. of bicarbonate-Ringer's solution) as they were cut from the organ, in order to insure uniform sampling. All slices were allowed to diffuse for the same length of time, before precipitation. For example, if incubation of one sample of tissue continued for 2 hours, the "initial" sample was allowed to diffuse for 2 hours at 1°C. At this temperature no lactic acid formation took place in liver tissue. When incubation was to be carried out in nitrogen, the air was removed from the flask by means of an electric pump, and the flask washed out two or three times with pure nitrogen before the final filling. In some experiments oxygen was bubbled through the Ringer's solution in which the tissue was suspended for about 30 minutes before replacing with nitrogen in the usual way. According to Rosenthal (1928, 1929,[1] 1929[2]) this preliminary oxygen treatment causes

an increased lactic acid formation when the tissue is subsequently incubated in nitrogen. The results show that sliced liver produces more lactic acid than hashed or finely ground liver subjected to the same treatment. The smallest increases in lactic acid were obtained with finely ground liver. The increase during the period of incubation of slices is from 2 to 8 times that obtained with hash.

Striated muscle, on the other hand, formed lactic acid to about the same extent, whether the tissue was ground fine or sliced. In Experiment 49 the muscle was sliced the long way of the fibers instead of across them to lessen the damage to the individual cells. These observations with regard to liver and muscle hash and slices suggest that the lactic acid formation of liver is much more dependent upon undisturbed tissue structure than is that of muscle. It should be realized, in considering the values of muscle lactic acid presented, that since no extra precautions were used in removing muscles from the animals, the initial figures are very high and do not represent resting muscle values.

The Effect of Freezing

Since grinding had been shown to have an inhibitory effect on the lactic acid formation in liver, it was thought that freezing might cause further inhibition of this activity. Experiments were, therefore, performed in which the liver was frozen and pulverized or was sliced and then frozen previous to the period of incubation. The data are given in Table V. In the Experiments 14 to 38 liver was powdered in a mortar after freezing for about 10 minutes with liquid air or carbon dioxide snow. Liver prepared in this way formed little or no lactic acid on incubation at 37°. Giragosintz and Olmsted (1930) have shown that considerable errors may arise when tissue is frozen with liquid air, owing to the loss of water from the tissue. This seems to be especially true when small amounts of tissue are used. There are probably no significant errors in the present experiments due to this cause. In Experiment 35 (Table III) a comparison was made between tissue cooled in a beaker immersed in salt and ice and only slightly frozen at the edges, and tissue frozen with

liquid air and pulverized. The ether extraction procedure demonstrated that both portions contained the same concentration of lactic acid.

TABLE V

THE EFFECT OF FREEZING ON THE LACTIC ACID FORMATION IN LIVER TISSUE OF RABBITS

Results are Expressed as Mgs. Lactic Acid per 100 Grams of Fresh Liver

EXPT. NO.	TEMP. DURING PREPARATION	INITIAL	FINAL			
			FREEZING MEDIUM	GROUND	SLICED	INCUBATION
14	3	62*	liquid air	70*		Vacuum 120′ at 25°
18	3	48*	liquid air	53*		Vacuum 240′ at 25°
19	3	79*	CO_2 snow	85*		Vacuum 120′ at 25°
20	3	104*	CO_2 snow	100*		Vacuum 120′ at 25°
21	3	50*	Liquid air	62*		Vacuum 120′ at 42°
29	3	75	liquid air	76		Vacuum 120′ at 25°+ 120′ at 37°
35	3	99	liquid air	88		Vacuum 120′ at 25°+ 120′ at 37°
38	3	75	liquid air	78		Vacuum 120′ at 25°+ 120′ at 37°
50	25	111	liquid air		120	30′ O_2 120′ N_2 all at 37°
			NaCl—ice		168	30′ O_2 120′ N_2 all at 37°
51	25	128	liquid air		127	30′ O_2 120′ N_2 all at 37°

* Exact condition of animal unknown and lactic acid determined on copper filtrates.

Since it was thought possible that the complete inhibition of lactic acid production in the above experiments might be due solely to the fine pulverization made possible by freezing, some experiments were performed in which liver slices, frozen, but not pulverized, were used. The results show that freezing with liquid air may completely stop the formation of lactic acid. In Experiment 51 the liver slices which had been frozen, formed no lactic acid on incubation, while unfrozen slices from the same tissues produced 278 mg. of lactic acid during the incubation period (see Table IV).

When a temperature only a few degrees below zero was used for freezing, the activity was not checked completely by 10 minutes' freezing, but was greatly inhibited. Experiment 50 illustrates this. Some of the slices were spread out on the bottom of a glass dish which was then immersed in a mixture of ice and salt until the tissue had been frozen for about 10 minutes. The upper surface of the slices appeared to be frozen solid so that the slight lactic acid formation was apparently not due to cells which escaped freezing.

It is concluded from the experiments thus far described, that any treatment which damages the structure of liver tissue decreases its capacity for lactic acid production. This conclusion may explain the results of numerous experiments involving liver autolysis, [Sevringhaus, Koehler, and Bradley (1923), Rona and associates (1923), (1925)] in which the liver was usually ground to a pulp and treated with an antiseptic and was sometimes previously frozen. In these experiments there was usually only a small amount of lactic acid produced over a period of several days.

The loss of power, by liver, to form lactic acid after being frozen by liquid air was overlooked by Simpson and Macleod (1927). In order to compare carbohydrate metabolism of liver and muscle each tissue was frozen with liquid air, pulverized, and then allowed to stand at room temperature in salt solution. They found that, whereas glycogen disappeared from both tissue suspensions, lactic acid was formed by the muscle alone. The liver suspension formed reducing sugar but no lactic acid. The conclusion was drawn that muscle functions by forming lactic acid and no sugar whereas liver forms sugar and no lactic acid.

The present investigations demonstrate that the lack of formation of lactic acid by liver in the experiments of Simpson and Macleod was probably due to the fact that the lactic acid forming system had been destroyed by freezing and grinding. While the main function of the liver in vivo may be to form sugar from its glycogen stores it is nevertheless true that liver may form considerable amounts of lactic acid.

The lactic acid forming enzyme system is much more stable in muscle than in liver. It is, therefore, possible that the enzymes involved are fundamentally different. The enzymes of muscle vary in stability. Meyerhof has shown (1926[2]) that the esterifying mechanism of frog muscle is destroyed by standing 15 minutes at 38° or overnight in the ice box. Laquer's experiments on striated muscle (1922) demonstrated that freezing in liquid air stopped the formation of lactic acid from added glucose but not from added glycogen. Laquer pointed out that the "genuine" lactic acid production of muscle was not inhibited by freezing since equal amounts of lactic acid were formed, without addition of carbohydrate, in previously frozen and unfrozen muscle hash.

That other tissues are similar to liver and unlike striated muscle with respect to the relation of cell structure to lactic acid production is suggested by studies such as the following: Warburg, Posener and Negelein (1924) have shown that freezing in liquid air destroys the glycolytic power of carcinoma, and also terminates its life processes. After this treatment the tissue is no longer transplantable. Barr, Ronzoni, and Glaser (1928) observed that hashing diminished, and that grinding and freezing completely stopped the glycolytic process in tumor tissue. Ronzoni (1928) found that freezing prevented lactic acid formation in the smooth muscle of the chicken. Indeed, it seems that the lactic acid production of striated muscle is rather peculiar in its relative independence of tissue structure. Further evidence lies in the fact that lactic acid formation has been demonstrated in muscle juice (Embden, Karlbach, and Engel (1912[2]) and Davenport, and Cotonio (1927), and that, as Meyerhof (1926[1]) has shown, an extract of striated muscle can produce lactic acid from added glycogen at practically the same rate as that of the

spontaneous lactic acid formation of minced muscle. Barr, Ronzoni, and Glaser (1928) attempted to prepare such an extract from tumor tissue, but were unable to show any lactic acid formation.

A Comparison of Lactic Acid Formation in the Presence and Absence of Oxygen, and the Effect of Preliminary Oxygen Treatment

Lactic Acid Formation in the Presence and Absence of Oxygen

As early as 1907, Fletcher and Hopkins (1907) observed that excised whole muscle produced lactic acid spontaneously in nitrogen, to a smaller extent in air, and practically not at all in pure oxygen. Since that time, literally hundreds of papers have been published on the lactic acid formation of different tissues under various conditions *in vitro*. In general, the spontaneous lactic acid formation is studied anaerobically, as under these conditions the greatest lactic acid formation is likely to occur since oxidative removal of this product cannot take place. In view of the results in the literature, it was rather surprising to find that we could show little difference in aerobic and anaerobic lactic acid formation in liver.

In Table VI, Experiments 22, 23 and 37 were carried through in the same way as those described in the section on liver hash. One flask, however, was left open to the air. It seems clear from these experiments that the presence of oxygen has little little effect on the formation of lactic acid in liver hash. In Experiments 41 to 54 bicarbonate-Ringer's solution was used as previously described. It will be noted that some of the tissues were worked up at room temperature without previous cooling. With this preparation, ground liver produced lactic acid to about the same extent (that is, the initial content about doubled in 2 hours) as in the first group of experiments on liver hash.

Since it has been shown that intact tissue structure is important for lactic acid formation in liver, tissue slices were used instead of hash in a few experiments. These experiments, comparing the lactic acid formation in the presence and absence

TABLE VI

LACTIC ACID FORMATION IN LIVER* IN THE PRESENCE AND ABSENCE OF OXYGEN, AND THE EFFECT OF PRELIMINARY OXYGEN TREATMENT
Results are Expressed as Mgs. Lactic Acid per 100 Grams of Fresh Liver

EXPT. NO.	TEMP. DURING PREPARATION	SUSPENSION FLUID	INITIAL	FINAL		ATMOSPHERE
				HASHED	SLICED	
22	3	2 per cent bicarbonate	44	79 72		Vacuum Air
23	3	2 per cent bicarbonate	42	122 113		Vacuum Air
37	3	2 per cent bicarbonate	97	193 195		Vacuum Air
41	25	Bicarbonate-Ringers	53	136		30′ O_2+ 120′ N_2
42 (Rats)	25	Bicarbonate-Ringers	176	286 278 290		150′ Vacuum 30′ O_2+ 120′ N_2
43	25	Bicarbonate-Ringers	132		450 579	150′ N_2 30′ O_2+ 120′ N_2
44	3	Bicarbonate-Ringers	115	157† 161†	481	150′ Vacuum 30′ O_2+ 120′ N_2
52	25	Bicarbonate-Ringers	117	177† 168†	218 225	120′ Air 120′ N_2
54	25	Bicarbonate-Ringers	120		141 186 189 229	30′ O_2 60′ O_2 60′ N_2 30′ O_2+ 60′ N_2

* Rabbit liver except where otherwise noted.
† Liver ground very fine.

of oxygen indicated that oxygen did not inhibit the process. To exclude the possibility that the thickness of slices usually used in our experiments might be too great for lactic acid studies of this sort a control experiment was planned to determine whether or not thinner slices would yield results different from those

previously obtained. For this experiment the liver of a young rabbit (4 months old) was cut into slices, which were at once placed in a small volume of bicarbonate-free Ringer's solution. A number of slices were cut in the usual way, and about 50 additional slices were cut according to Warburg's (1923) specifications (average thickness, about 0.2 to 0.3 mm.). When enough slices had been cut, all were removed from the Ringer's solution. The very thin slices were placed on glass needles which had been fused on a glass spiral as suggested by Warburg, and were placed in 15 cc. of bicarbonate-Ringer's solution. The thicker slices were divided into two approximately 5 gram portions and each portion placed in 100 cc. of bicarbonate-Ringer's solution. For the "initial" lactic acid value, one of these portions was allowed to stand at about 1°C for an hour. The other portion of thick slices, as well as the thin slices, were kept at 37.5°C for an hour, during which time pure nitrogen was bubbled through the solutions. At the end of the experimental period, slices were removed from the various solutions, and weighed after drying at 110°. The lactic acid was of necessity determined on the solutions only. The initial lactic acid proved to be 363 mg. per 100 grams of dry tissue (approximately 90 mg. per cent fresh tissue). The solution in which the thick slices had been incubated showed a lactic acid content corresponding to 1027 mg. per 100 grams of dry tissue (approximately 257 mg. per cent fresh tissue), and that in which the thin slices had been suspended was 1021 mg. per 100 grams dry tissue (approximately 255 mg. per cent fresh tissue). This shows a lactic acid increase in both thick and thin slices amounting to about 664 mg. per cent per hour, or 166 mg. per cent as calculated for fresh tissue. This falls within the range of Rosenthal's (1929[2]) figures, although it is slightly lower than the average. The experiment signifies that the thickness of the slices generally used in our experiments is probably permissible and adequate for the study of lactic acid formation in nitrogen.

To return to Table VI, it is difficult to explain the results such as those of Experiment 54, since it would seem that within an hour's time enough oxygen would diffuse into the slices to retard lactic acid formation or even cause a resynthesis of higher

carbohydrate. Since this was evidently not the case, it is possible that the slices usually employed were too thick for the study of the aerobic production of lactic acid, as most investigators who use the Warburg manometric technique in their experiments find greater lactic acid production anaerobically than aerobically. Warburg, Posener, and Negelein (1924) found that for liver in the so-called "stationary condition" $Q\frac{O_2}{CO_2}$* was 0.6, which amounts to about 60 mg. of lactic acid per 100 gm. of fresh tissue per hour (taking 75 per cent as the water content of liver) and $Q\frac{N_2}{CO_2}$ was 3.3 which is equivalent to about 330 mg. per 100 grams. Rosenthal and Lasnitzki (1928) found for liver of normal rats, a variable aerobic lactic acid formation of about 80 mg. per hour and an anaerobic lactic acid production of about 114 mg. per hour. Rosenthal's later work on liver (1929[1], 1929[2]) shows that without added glucose, the anaerobic lactic acid formation of about 120 to 500 mg. per 100 grams of fresh tissue per hour. Minami (1923) obtained lactic acid formation in liver in the presence of air, amounting to 100 mg. per 100 grams of fresh tissue per hour, as determined by the v. Fürth-Charnass method, following ether extraction. He obtained similar results with Warburg's manometric method, both in air and nitrogen when added glucose was used as the substrate. It was observed by Tamiya (1927), that there was a decrease in both aerobic and anaerobic glycolysis with increasing age of the animal from which the tissue was taken. Hawkins (1928) found, however, that in rat liver, while the anaerobic glycolysis decreased with age, the aerobic glycolysis increased. In the above experiments, those figures obtained by the manometric method are for strips of tissue suspended in glucose-containing Ringer's solution as described by Warburg, except where otherwise stated. With the possible exception of Minami's work, then, the conclusion has

---

* Q_{CO_2} = cmm. CO_2 formed per hour per mg. of dry tissue. The CO_2 is a measure of the lactic acid formed.

been in general, that anaerobic lactic acid formation is greater than the aerobic lactic acid production in liver. Hawkins found practically the same amount of glycolysis aerobically and anaerobically in the livers of very old rats, but this could hardly be used to explain the results presented here, for the rabbits employed were young animals.

The Effect of Preliminary Oxygen Treatment

Rosenthal and Lasnitzki (1928) and Rosenthal (1929[1], 1929[2]) have demonstrated that if liver slices are agitated in glucose-free Ringer's solution in the presence of oxygen at 37.5° and then allowed to glycolyse under anaerobic conditions, the amount of lactic acid formation which takes place per hour is greater than without the preliminary aerobic period. We were unable to demonstrate any difference between the lactic acid formation of liver hash with and without the aerobic period. In carrying out the experiments, liver hash was incubated in the presence of oxygen for a short time (15–30 minutes) before the 2-hour period was begun. The resulting lactic acid formation was not materially greater than when the hash was kept in nitrogen for the entire incubation period.

Similar experiments were performed with liver slices, and here results are irregular. Experiment 54 was designed to show definitely, the lactic acid formation after preliminary oxygen treatment as compared with that obtained without oxygen treatment. It will be noted that the same increase in lactic acid is obtained with pure oxygen after one hour at 37.5° as with pure nitrogen (66–69). If the value 141, for lactic acid after ½-hour in oxygen, is subtracted from 229, for ½-hour in oxygen plus one hour in nitrogen, an increase of 88 mg. per cent is obtained for the period in nitrogen. In this case, the lactic acid increase was augmented 27 per cent by preliminary treatment with oxygen. Experiment 43 also indicates that there may be increased activity due to preliminary treatment with oxygen. Unfortunately, the data are not complete, but it will be seen that there is less lactic acid formation (312 mg. per cent) when nitrogen was used (447). This indicates an "extra fermentation" as it is

designated by Rosenthal, of approximately 40 per cent as measured over a period of 2½ hours.

It is rather difficult to compare results since those of Rosenthal were obtained by the manometric method and since the technique employed was not exactly the same, but in general the extra fermentation reported is very much greater than was obtained here. The average extra fermentation of liver slices in glucose-free Ringer's solution, calculated on the basis of $Q_M^{N_2}$ values for the first and second hours of incubation was 230 per cent. Rosenthal has recently been able to check his results obtained by the chemical method with the v. Fürth-Charnass-Clausen chemical method when salivary gland was used. It is possible that individual differences in the animals used may account for the disagreement between Rosenthal's results and those of the experiments here presented. The great likelihood of frequently obtaining erratic results in this type of work is generally conceded.

Lactic Acid Formation From Added Carbohydrate In Liver Slices From Fasted Animals

The precursor of lactic acid in striated muscle, whether directly or indirectly, is generally conceded to be glycogen. Meyerhof has shown (1926[1]) that lactic acid can also be formed in cell-free enzyme extracts of muscle from phosphate esters and even, under certain conditions, from hexoses. The mechanism for lactic acid formation in liver and also the precursor of lactic acid are as yet not definitely known.

The work of Minami, Warburg, and others indicates that lactic acid can be formed from glucose in liver. Rosenthal and Lasnitzki found (1928) however, that although most tissues formed lactic acid abundantly from added glucose, liver of growing rats did not seem to be able to utilize glucose in this way. Later (1929[1]) Rosenthal observed that lactic acid formation in liver slices was slightly increased over the spontaneous formation, but that the increase was so insignificant as to indicate that glucose could not be regarded as a source material for lactic acid formation.

In order to study the effect of added glycogen and glucose an

experiment was performed in which these two carbohydrates were added to separate portions of incubating sliced liver. All liver samples were from the same fasting rabbit. Besides glycogen from a commercial source, a sample of glycogen prepared from rabbit liver was used. For the preparation of this material, fresh liver was ground in 5 times its volume of cold 5 per cent trichloracetic acid and allowed to stand for about 2 hours. After removal of the precipitated protein by filtration, an equal volume of 95 per cent alcohol was added, and the resulting flocculent precipitate was allowed to settle. The supernatant alcoholic solution was removed by decantation, and the precipitate was dissolved in water and reprecipitated with alcohol. After it was washed several times with absolute alcohol, the product was dried in air, and finally in a desiccator. The resulting pure white powder which produced a red color with iodine, was supposed to be mainly glycogen. The amounts of material isolated suggest that the method may be modified for quantitative

TABLE VII

LACTIC ACID FORMATION IN SLICED LIVER OF A FASTING RABBIT IN THE PRESENCE OF VARIOUS MATERIALS
Results are Expressed as Mgs. Lactic Acid per 100 Grams of Fresh Liver

EXPT. NO.	INITIAL	Final 30′ O_2 + 120′ N_2			
		BICARB.-RINGER'S	BICARB.-RINGER'S 2 PER CENT GLUCOSE	BICARB.-RINGER'S 2 PER CENT RABBIT GLYCOGEN	BICARB.-RINGER'S 2 PER CENT COMMERCIAL GLYCOGEN
48 (5-day fast)	58	165	195	197	190

analysis. Lactic acid in control and experimental samples (Table VII) was determined after preliminary oxygen treatment and anaerobiosis as already described. The lactic acid increased by about 100 mg. per cent in the control, and by about 140 mg. per cent in all of the samples in which carbohydrate had been added. The actual increase in lactic acid formation due to

added carbohydrate, was about 30 per cent. The experiment with glucose falls in line with Rosenthal's inasmuch as it demonstrates that glucose does not, under the conditions of the experiment, increase the lactic acid formation of liver slices very markedly.The same is true of glycogen, both commercial and that prepared from rabbit liver. As the lactic acid formation probably occurs only in the intact liver cells it is surprising that the non-diffusible glycogen should cause any increase in the lactic acid formation.

SUMMARY

1. A comparison of methods for the preparation of live tissue for lactic analysis is presented. Errors are shown tr. occur when the Schenk and copper-lime procedures are usedo Ether extraction was found to be more satisfactory.

2. Maximum lactic acid formation with liver is obtained when slices are incubated. Grinding reduces the subsequent lactic acid formation, and freezing may stop it entirely. Muscle hash and slices produce lactic acid with about equal rapidity.

3. Preliminary oxygen treatment has little effect on anaerobic lactic acid formation in thick slices of liver.

4. Glucose and glycogen increase the lactic acid formation in liver slices by about 30 per cent.

The writer wishes to thank Dr. D. Wright Wilson for many suggestions and criticisms he has given in the pursuance of this investigation.

BIBLIOGRAPHY

Barr, D. P., Ronzoni, E., and Glaser, G. (1928), *J. Biol. Chem. 80*, 331.
Clausen, S. W. (1922), *J. Biol. Chem. 52*, 263.
Cori, C. T. (1925), *J. Biol. Chem. 63*, 253.
Davenport, H. A., and Cotonio, M. (1927), *J. Biol. Chem. 73*, 463.
Embden, G., and Kraus, F. (1912[1]), *Biochem. Z. 45*, 1.
Embden, G., Karlbach, F., and Engel, H. (1912[2]), *Z. physiol. Chem. 45*, 45.
Embden, G. (1925), *Z. physiol. Chem. 143*, 297.
Fletcher, W. M., and Hopkins, F. G. (1907), *J. Physiol. 35*, 247.
Fahrig, C. (1927), *Z. Krebsforsch. 25*, 146.
Friedemann, T. E., Cotonio, M., and Shaffer, P. A. (1927), *J. Biol. Chem. 73*, 335.
Friedemann, T. E., and Kendall, A. I. (1929), *J. Biol. Chem. 82*, 23.
v. Fürth, O. and Charnass, D. (1910), *Biochem. Z. 26*, 199.
Girazosintz, G. and Olmsted, J. M. D. (1930), *Am. J. Physiol. 92*, 414.
Harada, Y. (1927), *Biochem. Z. 188*, 172.
Hawkins, J. A. (1928), *J. Gen. Physiol. 11*, 645.
Himwich, E. and Jacobson, S. (1927), *Proc. Soc. Exp. Biol. and Med. 25*, 53.
Hirsch-Kaufman, H. (1924), *Z. physiol. Chem. 140*, 25.
Irving, J. (1928), *Biochem. J. 22*, 1508.
Laquer, F. (1922), *Z. physiol. Chem. 122*, 26.
Loebel, R. (1925), *Biochem. Z. 161*, 219.
Meyerhof, O. (1926[1]), *Biochem. Z. 178*, 395.
Meyerhof, O. (1926[2]), *Biochem. Z. 178*, 462.
Minami, S. (1923), *Biochem. Z. 142*, 334.
Murphy, J. and Hawkins, J. (1925), *J. Gen. Physiol. 8*, 115.
Riesser, O. and Hansen, A. (1926), *Z. physiol. Chem. 161*, 149.
Rona, P., and Mislowitzer, E. (1923), *Biochem. Z. 140*, 517.
Rona, P., Mislowitzer, E., and Seidenberg, S. (1925), *Biochem. Z. 162*, 87.
Ronzoni, E. (1928), *J. Biol. Chem. 78*, lv.
Rosenthal, O. and Lasnitzki, A. (1928), *Biochem. Z. 196*, 340.
Rosenthal, O. (1929[1]), *Biochem. Z. 207*, 263.
Rosenthal, O. (1929[2]), *Biochem. Z. 211*, 295.
Schmitz, E., and Chrometzka, F. (1925), *Z. physiol. Chem. 144*, 196.
Sevringhaus, E. L. (1923), *J. Biol. Chem. 57*, 181.
Sevringhaus, E. L. Koehler, A. E., and Bradley, H. C. (1923), *J. Biol. Chem. 57*, 163.
Simpson, W. and Macleod, J. J. R. (1927), *J. Physiol. 64*, 255.
Tamiya, C. (1927), *Biochem. Z. 189*, 175.
Warburg, O. (1923), *Biochem. Z. 142*, 317.
Warburg, O., Posener, K., and Negelein, E. (1924), *Biochem. Z. 152*, 309.

WESTBROOK PUBLISHING COMPANY
WPC
PHILA.